Hair Follicle has a Novel Anagen-Specific Protein, mKAP13

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To identify the anagen hair follicle-specific proteins, we screened the cDNA library prepared from the murine skins of anagen phase by the differential hybridization technique. Fifty-four cDNA clones expressed specifically in anagen phase were isolated, and most were found to correspond to known proteins in the hair follicles. Alternatively, we isolated a cDNA clone encoding a novel protein that possessed an entire open reading frame of 501 base pairs. This protein with a molecular weight of 17.9 kDa has no specific motifs nor significant homology to proteins already reported, although it contains some direct repeats that are often observed in intermediate filament-associated proteins and has a similar amino acid composition as a member of them. Northern blot analysis demonstrates that the transcript of this protein is skin specific, and that it is present in mid- and late anagen but not in catagen, telogen, and early anagen phases. The transcript appears to be expressed specifically in the keratogenous zone of the cortical cells of hair follicles, as exhibited by *in situ* hybridization. Furthermore, immunohistochemical study confirms that the protein is distributed in the cytoplasms of the keratinizing cortical cells and is undetected in the completely keratinized ones. These results suggest that this protein can be identified as a new member of intermediate filament-associated proteins and is related to the keratinization of the cortical cell layer in mouse hair. Therefore, we have termed this novel protein mKAP13 according to the unified nomenclature. ***Key words: differential hybridization/hair cycle/mouse. J Invest Dermatol 111:804–809, 1998***

Hair is a keratinized tissue produced under the cycle of growth, regression, and regrowth. This cycle typically has three phases; in the anagen phase, the hair follicle is regenerated and a new hair grows; in the catagen phase, hair elongation ceases and the follicle regresses; and in the telogen phase, the follicle remains at rest. Within the anagen phase, six stages (anagen I–VI) have been further histologically identified (Chase *et al*, 1951).

Structurally, mouse hair is composed of a cuticle sheath, an inner cortex, and a central medulla, and its major structural proteins have been identified as keratins. Keratin proteins are derived from several multigene families and have been classified into hair keratin intermediate filament proteins and intermediate filament-associated proteins (IFAP) (Powell *et al*, 1991). IFAP have been thought to form the rigid hair shaft by a cross-linked network of the keratin molecules, being classified as high-sulfur proteins (HSp) (16%–30% cysteine), ultra-high sulfur proteins (UHSp) (>30% cysteine), and high-glycine/tyrosine proteins (HGTp) according to their amino acid composition (Powell *et al*, 1991). Recently, another new member of IFAP termed Hacl-1 has been identified and has been shown to lack a remarkably high content of any specific amino acid.

So far, several genes coding for IFAP have been isolated from human, sheep, rabbit, and mouse. These genes have been shown to be activated transcriptionally in accordance with the hair cycle by northern blot experiments. *In situ* hybridization studies have demonstrated that their transcripts are expressed in the keratogenous zone of the cortex and/or cuticle of the hair shaft (MacNab *et al*, 1990; Fratini *et al*, 1993; Powell *et al*, 1995). In addition, the transcript of serine-rich UHSp has been detected in the medulla and inner root sheath of anagen hair, and the upper layers of the epidermis (Wood *et al*, 1990).

The processes of cell proliferation and differentiation are strictly regulated by distinct genes expressed transiently in response to specific signals. Such genes have been identified by differential hybridization (Klar *et al*, 1992), subtractive hybridization (Kavathas *et al*, 1984), and RNA fingerprinting using arbitrarily primed PCR (Ralph *et al*, 1993). Hair follicles are skin components for which the developmental stage can be easily confirmed by light microscopy. Moreover, because mouse skin shows hair growth synchronized in waves covering large skin areas, we can extract an abundant amount of RNA in a specific stage of the hair cycle. These observations imply that mouse skin is a reasonable tissue to use for differential screening in order to isolate anagen hair follicle-specific genes. In this study, we identify a new member of IFAP, mKAP13, using this strategy. The characterization of mKAP13 and the localization of its transcript and protein are examined in detail.

MATERIALS AND METHODS

Animals and skin samples Syngeneic C57BL/6 female mice (purchased from Charles River, Yokohama, Japan), 1–45 d and 7–8 wk old, and BALB/C mice, 24–42 d old, were housed under 12 h light/dark cycles and fed *ad libitum*.

For library construction and screening, hair growth (anagen) was induced as previously described (Paus *et al*, 1990). Seven w-C57BL/6 mice with all back skin follicles in telogen were depilated with a mixture of wax and rosin. Dorsal skins were harvested at days 0 (telogen) and 6 (anagen) after follicle growth induction. All skin samples were confirmed to be in telogen or anagen (anagen III–IV) phases histologically by a light microscope.

Library construction and screening Poly A^+ RNA were isolated from the dorsal skins of three C57BL/6 mice in anagen or telogen phases, respectively, by guanidine thiocyanate solubilization and centrifugation over a CsCl, and by an oligo (dT)-cellulose column (Sambrook *et al*, 1989). Directional cDNA

Manuscript received 9 January, 1998; revised June 23, 1998; accepted for publication July 24, 1998.

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Abbreviations: HGTp, high-glycine/tyrosine protein; HSp, high-sulfur protein; IFAP, intermediate filament-associated protein; UHSp, ultra-high sulfur protein.

library was constructed in λZAPII (Stratagene, La Jolla, CA) from poly A^+ RNA of anagen phase skin following the manufacturer's protocol. The cDNA library was plated and two replica filters were made for each plate. Duplicate filters were screened with radiolabeled first-strand cDNA probes derived from poly A^+ RNA of anagen and telogen phase skin. To label cDNA, poly A^+ RNA (2 μg) was incubated in 20 μl of 50 mM Tris (pH 8.3), 10 mM $MgCl_2$, 150 mM KCl, 1.0 mM dATP, 1.0 mM dGTP, 1.0 mM dTTP, 100 μCi of [^{32}P]dCTP (3000 Ci per mmol), 100 μg oligo(dT) per ml, 10 mM dithiothreitol, 10 U of RNasin (Promega, Madison, WI), 20 U of MMLV reverse transcriptase (GIBCO BRL, Gaithersburg, MD) for 60 min at 37°C (Klar *et al*, 1992). The unincorporated nucleotides were removed by chromatography using Sephadex G-50 column (Pharmacia, Uppsala, Sweden). Approximately 1×10^6 recombinant phages were plated and screened. Hybridization was performed at 42°C with two different probes (2×10^6 cpm per ml in hybridization solution), respectively, and followed by washing with 0.1 × sodium citrate/chloride buffer (150 mM NaCl, 15 mM trisodium citrate), 0.1% sodium dodecyl sulfate at 50°C (Sambrook *et al*, 1989). After two rounds of additional screening, the phages hybridized selectively with cDNA probe of anagen phase skin were isolated, and their cDNA inserts were excised directly as Bluescript plasmid (Stratagene) following the manufacturer's protocol.

DNA sequence and analysis The nucleotide sequences of the cDNA inserts were determined by the dideoxy chain-terminal method (Sanger *et al*, 1977) using double-stranded DNA as a template for BcaBEST DNA polymerase (Takara, Kyoto, Japan). The nucleotide sequence of the entire coding region was determined by sequencing both strands. Searching for the known sequences was performed with the GenBank database.

Northern blot analysis For northern blot analysis, total RNA were isolated using Isogen (Nippongene, Tokyo, Japan) from the back skin sections that were excised from each three C57BL/6 mice ranging in age from 1 d to 45 d and the various tissues according to manufacturer's recommendations. Ten micrograms of total RNA was electrophoresed in 1.5% formaldehyde gel with RNA molecular weight markers and transferred to nitrocellulose filter as previously described (Thomas, 1983). Full-length *mKAP13* cDNA was labeled with ^{32}P by a hexanucleotide random priming method (Feinberg and Vogelstein, 1983), and used as a probe. Hybridization and wash were carried out under essentially the same conditions as described above. Filters were subsequently rehybridized with the glyceraldehyde-3-phosphate dehydrogenase probe or the ^{32}P-end-labeled oligonucleotide (5′-TGGTCACCATGGTAGGCACGGCG-ACTACCATCGAAAGTTGAT) derived from 18S rRNA (Raynal *et al*, 1984).

Quantitation of mRNA levels Quantitation of the specific mRNA transcripts detected by northern blot was determined by BioImaging Analyzer BAS 2000 (FUJIX, Tokyo). All values were normalized for the same amount of RNA by determining the relative level of 18S rRNA in each sample. Furthermore, each transcript level was normalized to the maximum level of the transcript observed at day 9.

***In situ* hybridization** BALB/C mice, aged between 24 and 42 d, were sacrificed and the dorsal skins were taken parallel to the paravertebral line to obtain longitudinal hair follicle sections. The skin samples were fixed in 4% paraformaldehyde and embedded in paraffin according to the standard procedures. Five micrometer sections were mounted on silane-coated glass slides.

For the production of the anti-sense and sense RNA probes, a *mKAP13* cDNA clone covering the 5′ noncoding region, complete coding region, and 3′ noncoding region was linealized with *Eco*RI and *Xho*I, respectively. ^{35}S-UTP-labeled RNA probes were synthesized from the T7 and T3 promoter with RNA transcription kit (Stratagene).

In situ hybridization analysis was performed according to the method described previously (Powell and Rogers, 1990). Briefly, deparaffinized and deproteinized sections were acetylated in acetic anhydride solution and then dehydrated. Hybridization was performed at 55°C for 22 h with ^{35}S-labeled RNA probes at 2×10^7 cpm per ml. After treatment with RNase A and wash in 0.1 × sodium citrate/chloride buffer, sections were dipped into Kodak NTB-2 emulsion, exposed at 4°C for 21 d. Tissue sections were counterstained with hematoxylin.

Antibody and immunoblotting Monospecific antibody to mKAP13 was produced in rabbits by immunizing with a synthetic peptide, CGSSYPNNVFY-STDLQTPIT, corresponding to unique amino acid sequences. The immunization protocol was as previously described (Roop *et al*, 1984). The antibody was purified by affinity chromatography using the synthetic peptide coupled to activated Sepharose (Brinkley *et al*, 1980). For immunoblotting, the back skin of 36 d old BALB/C mice was dissolved in a sample buffer containing 2% sodium dodecyl sulfate, reduced by boiling with 5% β-mercaptoethanol, the sample (30 μg) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins in the gel were transferred onto nitrocellulose filter. Filter was preblocked in Tris-buffered saline [20 mM Tris (pH 7.5), 150 mM NaCl] containing both 0.1% Tween 20 and 10% non-fat milk, and washed with Tris-buffered saline containing 0.1% Tween 20. Primary antibody was used at 0.02% (vol/vol), and detection was performed with 0.1% horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako, Copenhagen, Denmark) in Tris-buffered saline with 0.1% Tween 20 and 10% non-fat milk by the enhanced chemiluminescence method (Amersham, Arlington Heights, IL).

Immunohistochemical analysis Paraffin sections of 29–42 d old BALB/C mice were deparaffinized and incubated sequentially with blocking serum,

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                                                        GAACATTCTCCTTATCCCATCCTCAAACTCCTAGCTCCTCTCAAC   45
ATG TCC TGC AAC AGC TGC TCT GGA ACT TTC TCC CAG TCC TTT GGG GGC CAA CTG CAG TAT  105
M   S   C   N   S   C   S   G   T   F   S   Q   S   F   G   G   Q   L   Q   Y

CCG ATC TCT TCA TGC GGT TCC TCC TAC CCC AAC AAC GTC TTC TAC AGC ACT GAC CTC CAA  165
P   I   S   S   C   G   S   S   Y   P   N   N   V   F   Y   S   T   D   L   Q

ACT CCC ATC ACC CAC CAG CTG GGC TCT TCT CTT CAC AGT GGG TGC CAG GAA ACC TTC TGT  225
T   P   I   T   H   Q   L   G   S   S   L   H   S   G   C   Q   E   T   F   C

GAG CCC ACC AAC TGC CAG ACA GCC TAT GTG GTC TCC AGA CCC TGC CAG AGG CCT TTC TAC  285
E   P   T   N   C   Q   T   A   Y   V   V   S   R   P   C   Q   R   P   F   Y

AGT CAG AGG ATT CGA GGG CCC TGC AGG CCC TGC CAG TCA ACT TTC TCG GGA TCC CTG GGA  345
S   Q   R   I   R   G   P   C   R   P   C   Q   S   T   F   S   G   S   L   G

TTT GGT TCC AGG GGT TTC CAG TCT TTT GGC TGT GGC TAC CCA TCC CAG GGC TTT GGA TCC  405
F   G   S   R   G   F   Q   S   F   G   C   G   Y   P   S   Q   G   F   G   S

CAT GGT TTC CAG TCA GTA GGA TGT GGT ACC CCT ACT TTC TCA TCC CTA AAT TGT GGA TCC  465
H   G   F   Q   S   V   G   C   G   T   P   T   F   S   S   L   N   C   G   S

AGC TTT TAC CGC CCA ACC TGC TTC TCT ACC AAA AGC TGC CAG TCT GTT TCT TAT CAG CCA  525
S   F   Y   R   P   T   C   F   S   T   K   S   C   Q   S   V   S   Y   Q   P

ACC TGT GGG ACT GGC TTC TTC TGA TCTCTATTGGGGAAAATTAGAAATGTAAAAGGTGCCTACTACCTACT  596
T   C   G   T   G   F   F   *

GTGTTAAGAGCCGTGCTGTCTTCAGAGTTTCTATATTAGTCTAGAGATTTGACTTCTACTCATGAGTTCCTGTAATTGG  675
AAAACGTATTTGATTAGAGAAAGATGCTAAATTGTTTTCTTGTATATTATAGGTATGCAAAAATCAAGCATAATTCTTT  754
CTTCGCATATCTCTTAGTAATTACTCAGACTTCCGGTCTTGATTTTTGCAAAGAGGAAGGTTGTAAGTTTAAATAAAGA  833
TATGCAACTGG                                                                       844
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Figure 1. Nucleotide sequence of *mKAP13* and deduced amino acid sequence of the protein product. Nucleotides are numbered consecutively at the right-hand side. The derived amino acid sequence appears below the nucleotide sequence. Polyadenylation signal is indicated by a double line. Three kinds of direct repeats are underlined, shaded, and boxed, respectively. The nucleotide sequence was registered in DDBJ, EMBL, and GenBank databases, and its accession number is D85925.

primary antibody, biotinylated second antibody, and streptoavidin-peroxidase following the manufacturer's protocol (Nichirei, Tokyo). Primary antibodies, affinity-purified anti-mKAP13 antibody and preimmune serum, were diluted 1:10,000 in phosphate-buffered saline and incubated overnight at 4°C in a humidified chamber. Antibody binding was visualized with diaminobenzidine and the sections were then counterstained with hematoxylin.

RESULTS

Isolation of cDNA clones expressed specifically in murine skin of the anagen phase Approximately 1×10^6 independent plaques of the cDNA library prepared from the murine skins of the anagen phase (anagen III–IV) were screened differentially with cDNA probes of the anagen and telogen phases. To isolate cDNA clones specific to the anagen phase, we selected the clones positive to the cDNA probe of the anagen phase and negative to that of the telogen phase. After three rounds of purification, 54 cDNA clones were isolated. Of these, 52 clones were shown through a screen of the GenBank database to encode known proteins corresponding to HSp (Frenkel *et al*, 1989; Powell *et al*, 1993), UHSp (MacNab *et al*, 1989), HGTp (Kuczek and Rogers, 1987; Fratini *et al*, 1993; Aoki *et al*, 1997), trichohyalin (Fietz *et al*, 1990), ribosomal protein L35a and S6 (Kuzumaki *et al*, 1987; Lalanne *et al*, 1987), and elongation factor 1-α (Lu and Werner, 1989). Alternatively, two cDNA clones were shown to encode novel proteins. Particularly, one clone contained a complete open reading frame and was shown on the basis of its deduced amino acid sequence to encode a protein that has no specific motifs or significant homology with proteins previously described. This cDNA clone was termed *mKAP13* after the characterization of its spatial and temporal expression in hair follicles.

The predicted amino acid sequence of mKAP13 exhibits that it is a novel protein *mKAP13* cDNA was shown to contain a single long open reading frame that starts with a methionine codon at nucleotide 46 with a conventional translation initiation sequence (Kozak, 1986), and ends with a TGA stop codon at nucleotide 547 (**Fig 1**). Polyadenylation signal, AATAAA, is located at nucleotide 826 and presents 19 nucleotides upstream from the poly (A) RNA sequence. Translation of the open reading frame of its cDNA predicted a protein of 167 amino acids with a molecular weight of 17.9 kDa. Serine content of this protein was demonstrated to be high, i.e., 18.5%, although the cysteine, glycine, and tyrosine contents are not remarkably high, i.e., 8.9%, 12.5%, and 4.7%, respectively. In the deduced amino acid sequence, tetrapeptide (C/L)GSS appeared three times, and tetrapeptide RPCQ and tridecapeptide GFGS(R/H)GFQS(F/V)GCG appeared twice. Screening of the Genbank database with its nucleotide sequence showed partial homologies with sheep *HSp* (47% nucleotide homology covering 600 bp) (Frenkel *et al*, 1989) and mouse *UHSp* (48%, 890 bp) (MacNab *et al*, 1989). The deduced amino acid sequence of mKAP13, however, does not fit well with those of above two proteins, indicating that it is a novel protein. Kyte–Doolittle plots failed to reveal any hydrophobic domain in mKAP13 that would suggest the presence of a transmembrane domain or a hydrophobic leader sequence.

***mKAP13* transcript is expressed specifically in skin** To investigate the expression of *mKAP13* in mouse tissues, northern blot analysis was performed with the total RNA from various mouse tissues using the full-length cDNA of *mKAP13* as a probe. As shown in **Fig 2**, its transcript was detected only in the skin (day 9) whereas it was not present in the other tissues including the tongue, esophagus, and forestomach, all of which are constituted of stratified squamous epithelia like skin.

Northern blot experiment demonstrated that *mKAP13* mRNA species is ≈850 base long, which is nearly the same size as its isolated cDNA.

Transcript of *mKAP13* is expressed in the anagen phase during the hair cycle To clarify the levels of the expression of the *mKAP13* transcript during hair growth cycle, northern blot analysis was performed with the total RNA extracted from the back skin of mice ranging in age from 1 d to 45 d. The hybridization result is shown in **Fig 3**, and after normalization for variable RNA loadings, is graphed in **Fig 4**.

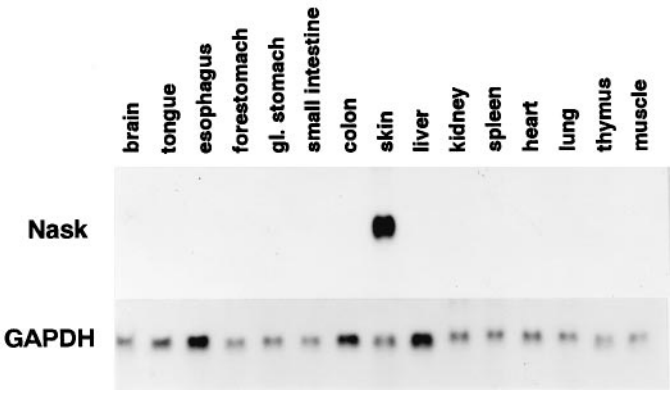

Figure 2. *mKAP13* transcript can be detected only in anagen skin by northern blot. Total RNA was extracted from various tissues of C57BL/6, separated by electrophoresis in 1.5% agarose, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled *mKAP13* whole-length cDNA. The blot was subsequently rehybridized with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. *mKAP13* transcript is detected only in the skin from 9 d old mice.

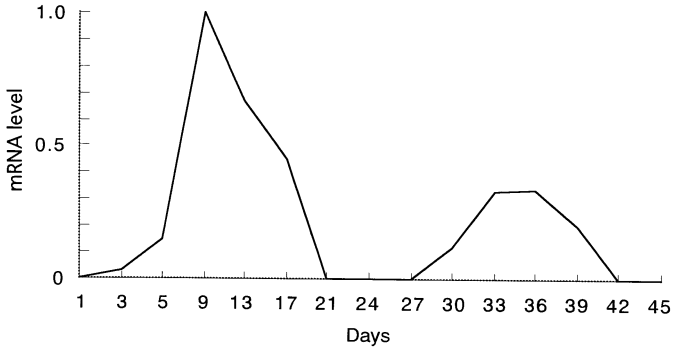

Figure 4. Quantitation of mRNA levels of *mKAP13*. Northern blot was scanned by BioImaging Analyzer BAS 2000 and the resultant values were normalized for equivalent amounts of RNA, moreover being normalized to the maximum level of the transcript observed at day 9.

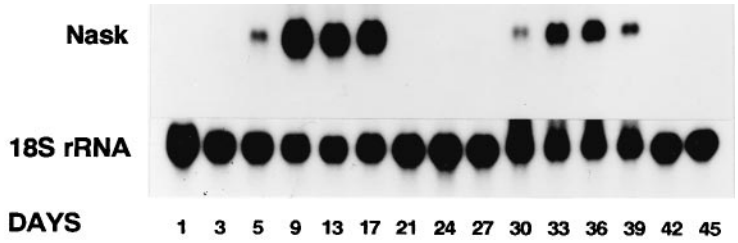

Figure 3. *mKAP13* transcript is expressed specifically in anagen phase during two hair cycles. Total RNA (10 μg) prepared from the back skin of C57BL/6 mouse ranging from day 1 to day 45 was electrophoresed and transferred to nitrocellulose filter. The filter was hybridized with ^{32}P-labeled *mKAP13* cDNA probe and followed by washing with 0.1 × sodium citrate/chloride buffer, 0.1% sodium dodecyl sulfate at 50°C. The blot was subsequently rehybridized with the ^{32}P-end-labeled oligonucleotide derived from 18S rRNA probe.

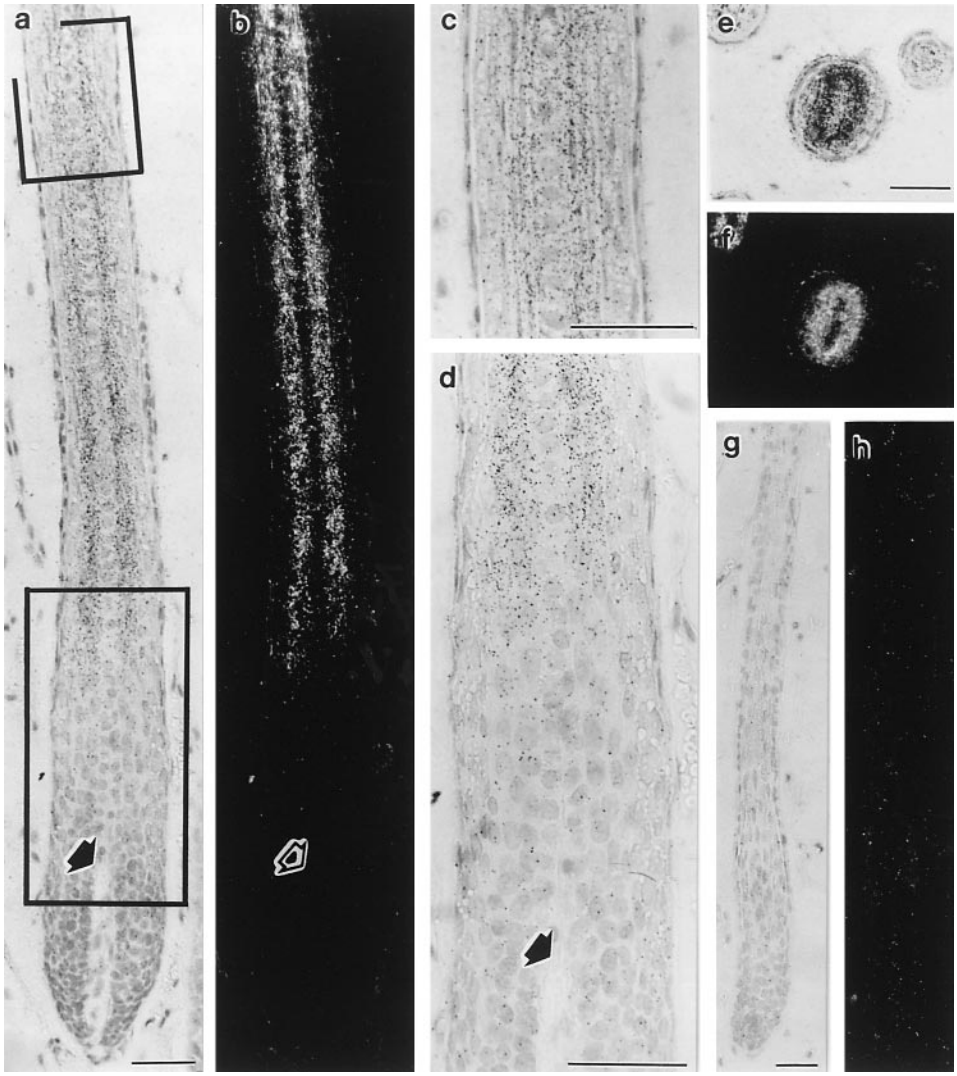

Figure 5. *mKAP13* transcript can be detected in the cortical cells in anagen VI but not in the catagen phase. *In situ* hybridization was performed in mouse skin at day 36 corresponding to anagen VI (*a–f*), and day 42 corresponding to catagen (*g*, *h*). (*a*, *c*, *d*, *g*) Brightfield and (*b*, *h*) darkfield views of longitudinal sections. (*e*) Brightfield and (*f*) darkfield views of transverse sections. (*c*, *d*) Higher magnifications of the boxed upper and lower parts of (*a*), respectively. *Arrows* indicate the approximate apex of the dermal papilla. *In situ* hybridization results in mouse skin at day 34 and 39 corresponding to anagen VI were fundamentally identical with those at day 36 (data not shown). Sense probe produced a random signal (data not shown). *Scale bars*: 50 μm.

This period represents two full cycles of hair growth. The expression of *mKAP13* mRNA follows the hair cycle fundamentally, increasing during the first anagen phase with its peak at day 9, and is not detectable during the catagen, telogen, and early anagen phases. Again, the levels of its mRNA accumulation increase during the anagen phase of the second hair growth cycle. In this second anagen phase, the mRNA accumulation seemed to reach a peak between day 33 and day 36, with levels being almost identical both days. The maximum gene expression was observed during the first hair cycle, with levels being approximately twice those at the peak during the second cycle.

mKAP13* transcript is expressed in the cortical cells in anagen V and VI** To determine the localization of *mKAP13* transcript, *in situ* hybridization was performed with BALB/C mouse, because the C57BL/6 mouse from which its cDNA was isolated had abundant melanin granules in the hair follicles, which seemed to disturb the analysis for the localization of transcript expressed (Aoki *et al*, 1997). The transcripts were undetected in mouse skin in telogen–anagen IV (day 24–29) (data not shown), expressed in anagen V, VI (day 32–39) (**Fig 5*a–f), and again disappeared in catagen phase (day 42) (**Fig 5*g*, *h***). In anagen VI, transcripts were detected predominantly in the keratogenous zone of the cortex region of the hair shaft, being distributed constantly from the cortical cells containing oval nuclei at a distance of about 120 μm above the apex of the dermal papilla to the ones containing flat nuclei before undergoing complete keratinization throughout this period. Transverse sections confirmed that no signal could be detected in the inner root sheaths (**Fig 5*e*, *f***). From our *in situ* hybridization study, it was not clearly determined whether the gene for this protein is expressed in the hair shaft cuticle cells, because the cuticle is composed of only a thin single cell layer. No expression was observed in the epidermis (data not shown) or any other hair follicle cells. Sense probe showed no positive signal under the same conditions (data not shown).

mKAP13 protein is distributed in the cytoplasms of the cortical cells in anagen V and VI To identify the distribution of mKAP13 protein in the hair follicles, antibody was raised in rabbits against a synthetic peptide corresponding to 20 unique residues. Western blot result using this antibody demonstrated the apparent molecular size of the protein to be ≈18 kDa, which is coincident with the size predicted from the amino acid sequence (**Fig 6**).

In the immunohistochemical study, this antibody showed no reaction with any skin components in catagen–anagen IV (data not shown). In anagen V and VI, it reacted with the cortex of the hair shaft (**Fig 7**). The immunostaining commenced at a distance of about 120 μm above the apex of the dermal papilla, reaching a peak in intensity at the middle portion of the hair shaft and gradually decreasing in accordance with keratinization, except for the reaction in the cortical cell projections into the medulla. The reaction was detected throughout the cytoplasm of the cortical cell (**Fig 7*b*, *d***). The matrix cells and the completely keratinized cells in the cortex displayed no reaction with this antibody (**Fig 7*c***). Furthermore, immunostaining was not observed in the other cells of the hair follicle and the epidermis, in which the transcripts were also not detected by *in situ* hybridization (data not shown). Preimmune serum showed no positive staining in the mouse skin (**Fig 7*e*, *f***).

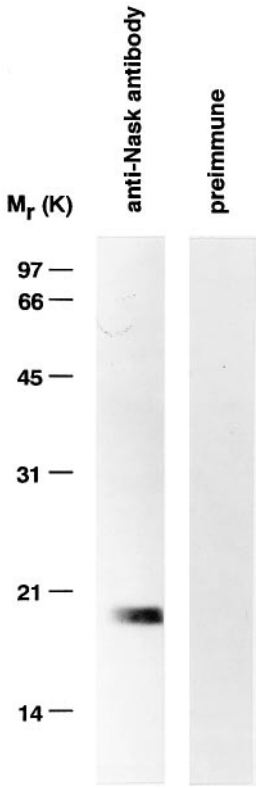

Figure 6. mKAP13 protein can be detected in mouse skin. Immunoblot was performed using affinity purified anti-mKAP13 antibody. As a negative control, preimmune serum was used. Note that an apparent band is only in anti-mKAP13 antibody.

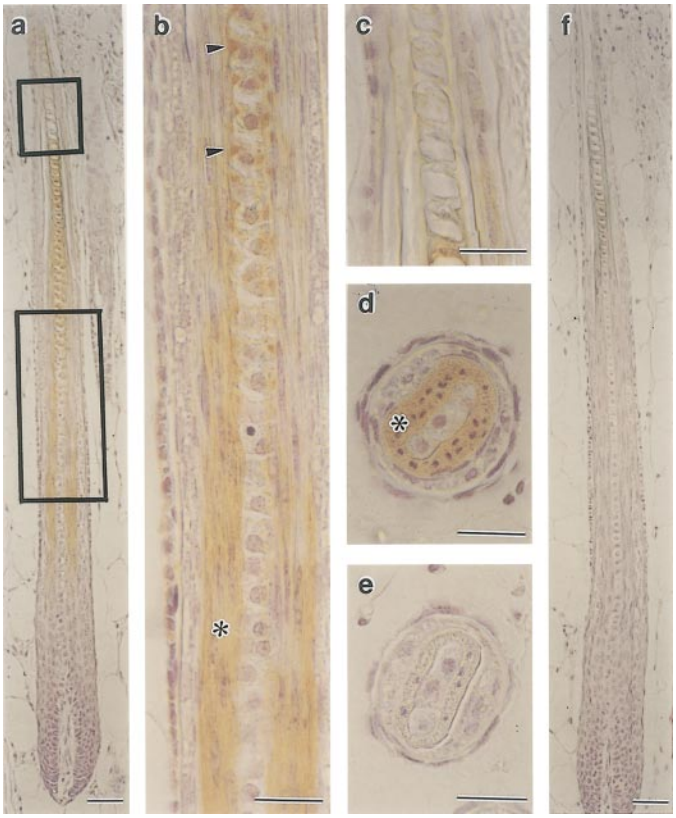

Figure 7. mKAP13 is distributed in the cytoplasms of the cortical cells in anagen VI. Paraffin sections from BALB/C mouse skin at day 36 corresponding to anagen VI were incubated with affinity purified anti-mKAP13 antibody (*a–d*) or preimmune serum (*e*, *f*), and counterstained with hematoxylin. (*b*, *c*) Higher magnifications of the boxed middle and upper parts of (*a*), respectively. The longitudinal (*a*, *b*) and transverse (*d*) sections demonstrate that the reaction is seen in the cortical cells of hair shaft (*asterisks*) and the cortical cell projections into the medulla (*arrowheads*). In the completely keratinized cortical cells, the immunostaining is not observed (*c*). The reaction at the control section (*e*, *f*) shows no labeling. *Scale bars:* (*a*, *f*) 50 μm; (*b–e*) 25 μm.

DISCUSSION

To isolate the genes expressed differentially between two distinct tissues or discrete developmental stages in the same tissue, differential screening techniques have often been performed, by which several novel genes have already been identified (Klar *et al*, 1992; Ralph *et al*, 1993). In this study, we attempted to identify the genes expressed in mouse anagen hair follicles by differentially screening skin in anagen and telogen phases. As a result, we isolated 52 cDNA clones encoding known proteins in addition to two clones encoding novel proteins. Most of these known proteins corresponded to trichohyalin, hair keratin intermediate filament proteins, and IFAP, all of which have been previously shown to be expressed at high levels in anagen hair follicles. This finding suggests that a number of the cDNA isolated by this strategy are expressed not in the epidermis but in hair follicles, implying that other cDNA clones coding for the novel proteins we isolated are also likely to be expressed in anagen hair follicles.

In this study, we identified a novel anagen skin-specific protein, mKAP13. A northern blot experiment demonstrated that its transcript is skin specific and not expressed in the other mouse tissues. Furthermore, the expression of the transcript in the skin was shown to follow the hair cycle fundamentally, similar to transcripts of *UHSp* (MacNab *et al*, 1989), *serine-rich UHSp* (Wood *et al*, 1990), and *HGTp* (Aoki *et al*, 1997). During a period ranging from day 21 to day 27, which covers catagen, telogen, and early anagen (anagen I–II) phases, *mKAP13* transcript seemed to either be not expressed or be expressed at very low levels, whereas its expression level reached a peak at the day corresponding to anagen VI. This suggests that mKAP13 is not related to the initiation of hair growth.

An *in situ* hybridization experiment exhibited that *mKAP13* transcript is expressed abundantly in late anagen phase as shown in the northern blot study. In anagen III–IV from which *mKAP13* cDNA has been isolated, its transcript was confirmed by northern blot analysis to be expressed faintly, whereas it is not observable at all in the *in situ* hybridization study. This discrepancy between the two studies most likely results from the very low expression of its transcript in the hair follicles of anagen III–IV. In anagen V–VI, its transcript was expressed predominantly in the keratogenous zone of the cortex region of the hair follicles. Furthermore, an immunohistochemical experiment showed that the protein is distributed in the keratogenous zone as well. These observations of the transcript and protein of mKAP13 being expressed in the same area of the cortex suggest that this protein is synthesized immediately after its transcription. Additionally, the finding that mKAP13 is distributed diffusely throughout the cytoplasmic area of the cortical cell is coincident with the notion that it is a cytoplasmic protein, as predicted from the amino acid sequence, and raises the possibility that it is localized between the hair keratin intermediate filaments, although their precise relation remains to be determined. The reaction in the cortex with the anti-mKAP13 antibody decreased in accordance with the keratinization, in spite of the constant expression of the transcript, suggesting that this protein is utilized soon after its synthesis following the keratinization process. These findings indicate that mKAP13 may be involved in the keratinization as a cytoplasmic protein in the cortical cells. A similar localization of the transcript in the keratogenous zone has been reported for some members of IFAP, such as UHSp (MacNab *et al*, 1990; Powell *et al*, 1995), HGTp (Fratini *et al*, 1993; Aoki *et al*, 1997), and Hacl-1 (Huh *et al*, 1994).

The nucleotide sequence of *mKAP13* shares a partial homology with sheep *HSp* and mouse *UHSp*, both of which are members of IFAP; however, its deduced amino acid sequence shows no significant homology with any proteins previously reported, including the above two proteins. mKAP13 has no remarkably high content of either

cysteine or glycine/tyrosine, suggesting that this protein does not belong to the groups of HSp, UHSp, and HGTp. Recently, Hacl-1 has been identified as a new member of IFAP and has been revealed to contain no predominant amino acids. Interestingly, mKAP13 has a similar mixture of amino acids as Hacl-1. Additionally, it is relatively enriched in serine, as observed in HSp, UHSp, and Hacl-1, and has several glutamine residues (9%), suggesting that it could well be a substrate for transglutaminase in the cortex. Furthermore, its unique feature is that it contains three kinds of direct repeats. Members of IFAP already identified have been reported to contain some direct repeats (Powell *et al*, 1995).

By differential screening, we isolated many cDNA clones expressed specifically in mouse skin during the anagen phase. Most of these cDNA were found to encode structural proteins of the hair follicles. In anagen hair follicles, growth factors and their receptors have been reported to be expressed (Du Cros *et al*, 1992). In this study, no cDNA coding for these proteins were isolated, which may result from the possibility that their transcriptions are not carried out differentially in mouse skin between the anagen and telogen phases, or that the transcripts of the structural proteins are expressed much more abundantly.

The novel protein we identified was determined to be expressed specifically in anagen hair follicle. Moreover, several lines of evidence obtained in this study suggest that it can be classified as a new member of IFAP. Therefore, we have used the term mKAP13 according to the unified nomenclature for IFAP (Rogers and Powell, 1993). In the future, the function of mKAP13 in the hair follicles should be elucidated further by producing transgenic or knockout mice using its gene.

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